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(54) Title: NOVEL HUMAN POTASSIUM CHANNEL SUBUNIT

(57) Abstract: The present invention is directed to novel human DNA sequences encoding Isk2, a potassium channel subunit, the protein encoded by the DNA sequences, vectors comprising the DNA sequences, host cells containing the vectors, and methods of identifying inhibitors and agonists of potassium channels containing the human Isk2 subunit.

WO 01/27246 A1

TITLE OF THE INVENTION
NOVEL HUMAN POTASSIUM CHANNEL SUBUNIT

CROSS-REFERENCE TO RELATED APPLICATIONS

5 Not applicable.

STATEMENT REGARDING FEDERALLY-SPONSORED R&D

Not applicable.

10 REFERENCE TO MICROFICHE APPENDIX

Not applicable.

FIELD OF THE INVENTION

15 The present invention is directed to a novel human DNA sequence encoding a potassium channel subunit, protein encoded by the DNA sequence, methods of expressing the protein in recombinant cells, and methods of identifying activators and inhibitors of potassium channels comprising the subunit.

BACKGROUND OF THE INVENTION

20 Voltage-gated potassium channels form transmembrane pores that open in response to changes in cell membrane potential and selectively allow potassium ions to pass through the membrane. Voltage-gated potassium channels have been shown to be involved in maintaining cell membrane potentials and controlling the repolarization of action potentials in many cells, *e.g.*, neurons, muscle
25 cells, and pancreatic β cells. Following depolarization, voltage-gated potassium channels open, allowing potassium efflux and thus membrane repolarization. This behavior has made voltage-gated potassium channels important targets for drug discovery in connection with a variety of diseases. As a result, many voltage-gated potassium channels have been identified and many cloned. They are distinguishable
30 by their primary amino acid sequences, tissue-specific patterns of expression, and by electrophysiological and pharmacological properties. For reviews of voltage-gated potassium channels see Robertson, 1997, Trends Pharmacol. Sci. 18:474-483; Jan & Jan, 1997, J. Physiol. 505:267-282; Catterall, 1995, Ann. Rev. Biochem. 64:493-531.

Many functional voltage-gated potassium channels are believed to be tetramers of four α subunits, each of which contains six transmembrane spanning segments. The α subunits making up a tetramer may be the same (in the case of homotetramers) or may be different (in the case of heterotetramers). The membrane-spanning α subunits making up the tetramers may sometimes be associated with additional, β subunits, which may alter the behavior of the tetramers.

In contrast, other types of voltage-gated potassium channels appear to have a somewhat different structure, containing additional types of subunits. In particular, the potassium channel that mediates part of the repolarization of cardiac action potentials is a heteromer of two different subunits, KvQLT1 and minK, and is responsible for a slowly activating, delayed-rectifier potassium current known as I_{Ks} (Sanguinetti et al., 1996, Nature 384:80-83).

KvQLT1 is a protein having six hydrophobic membrane-spanning α helices and a typical potassium channel signature sequence (Sanguinetti et al., 1996, Nature 384:80-83; Heginbotham et al., 1994, Biophys. J. 66:1061-1067). MinK is a small (129-130 amino acids) protein with a single transmembrane domain. (Takumi et al., 1988, Science 242:1042-1045; Swanson et al., 1993, Sem. Neurosci. 5:117-124). KvQLT1 alone can form an ion channel, but the presence of minK is necessary to produce a complex that has the slow gating kinetics, small unitary conductance, sensitivity to second messengers, and affinity for class III antiarrhythmic agents shown by native I_{Ks} channels (Takumi et al., 1988, Science 242:1042-1045; Goldstein & Miller, 1991, Neuron 7:403-408; Blumenthal & Kaczmarek, 1994, J. Neurosci. 14:3097-3105; Sanguinetti et al., 1996, Nature 384:80-83; Busch et al., 1997, Br. J. Pharmacol. 122:187-189; Sesti & Goldstein, 1998, J. Gen. Phys. 112:651-664; Tai & Goldstein, 1998, Nature 391:605-608; Barhanin et al., 1996, Nature 384:78-80).

Another type of delayed-rectifier potassium current is the I_{Kr} current. The I_{Kr} current is mediated by a potassium channel in which the protein h-erg (human ether-a-go-go related gene) is the pore-forming subunit (Sanguinetti et al., 1995, Cell 81:299-307; Curran et al., 1995, Cell 80:795-803). MinK may also co-assemble with h-erg to form the functional cardiac I_{Kr} channel (McDonald et al., 1997, Nature 388:289-292).

It is desirable to discover other, novel potassium channel subunits related to known subunits, especially those exhibiting restricted tissue expression.

Such novel subunits would be attractive targets for drug discovery and would be valuable research tools for understanding more about ion channel biology.

Following the discovery of the IsK2 gene by the present inventors, Abbott et al., 1999, Cell 97:175-187 reported the cloning of a gene called MiRP1,
5 which has the same amino acid sequence as the IsK2 protein disclosed herein.

SUMMARY OF THE INVENTION

The present invention is directed to a novel human DNA sequence encoding IsK2, a protein resembling the minK subunit of delayed-rectifier potassium
10 channels. The DNAs of the present invention comprise the nucleotide sequence shown as SEQ.ID.NO.:1 and positions 79-447 of SEQ.ID.NO.:1. Also provided are proteins encoded by the novel DNA sequences. The proteins comprise the amino acid sequence shown as SEQ.ID.NO.:2 as well as fragments thereof. Methods of
15 expressing the novel subunit proteins in recombinant systems are provided as well as methods of identifying activators and inhibitors of potassium channels comprising the subunit proteins.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows a DNA sequence encoding IsK2 (SEQ.ID.NO.:1).
20 The start ATG codon is at position 79-81; the stop codon is at position 448-450. Figure 1B shows the deduced amino acid sequence (SEQ.ID.NO.:2) of the IsK2 subunit.

Figure 2 shows an amino acid sequence alignment of IsK2 (SEQ.ID.NO.:2) and human minK (SEQ.ID.NO.:3).

25 Figure 3A-F shows *in situ* hybridization/immunohistochemical localization studies of IsK2 expression in human stomach. Figure 3A-C shows the same section of tissue. Figure 3A shows *in situ* hybridization with a ribonucleotide probe specific for mRNA transcripts from the IsK2 gene labeled with biotin and detected with Texas Red; cells expressing IsK2 mRNA appear red. Figure 3B shows
30 immunohistochemistry of the same cells as in Figure 3A, but this time visualized with an antibody specific for gastrin and detected with a FITC-conjugated secondary antibody. Cells expressing gastrin peptide appear green. It can be seen that the pattern of red staining in Figure 3A is the same as the pattern of green staining in Figure 3B, indicating that the same cells that express IsK2 also express gastrin. This

is confirmed in Figure 3C which shows the results of simultaneously staining the cells for both IsK2 and gastrin. In this case, the cells appear yellow (a combination of red plus green), again indicating that the cells express both IsK2 and gastrin. Figure 3D shows another field of stomach cells stained for both IsK2 and gastrin. Again the only stained cells appear yellow. Figure 3E shows another field of stomach cells stained for both IsK2 and somatostatin (using an antibody to somatostatin and detected with a FITC-conjugated secondary antibody). In this case, cells expressing IsK2 (stained red) are different from cells expressing somatostatin (stained green). Figure 3F shows another field of stomach cells stained for both IsK2 and substance P (using an antibody to substance P and detected with a FITC-conjugated secondary antibody). In this case, cells expressing IsK2 (stained red) are different from cells expressing substance P (stained green).

Figure 4A-F shows Northern blot analysis of the expression of IsK2 (Figure 4A), KvLQT1 (Figure 4B), KCNQ2 (Figure 4C), KCNQ3 (Figure 4D), KCNQ4 (Figure 4E), and h-erg (Figure 4F) in various human tissues.

Figure 5A-E shows double *in situ* hybridization localization studies of the expression of IsK2 and other potassium channel subunits in human stomach. The ribonucleotide probe specific for mRNA transcripts from the IsK2 gene was labeled with biotin and detected with Texas Red; cells expressing IsK2 mRNA appear red. mRNA transcripts from the other potassium channel subunits were visualized with FITC, and thus appear green. Cells co-expressing Isk2 and another subunit appear yellow (combination of red and green). Figure 5A shows the results using probes specific for Isk2 and KCNH2 (herg); yellow cells are not seen. Figure 5B shows the results using probes specific for Isk2 and KCNQ4; yellow cells are not seen. Figure 5C shows the results using probes specific for Isk2 and KCNQ1 (KvLQT1). Figure 5D is an enlargement of Figure 5C; many yellow cells are apparent; indicating co-expression of Isk2 and KCNQ1 in those cells and suggesting that Isk2 and KCNQ1 may co-assemble to form heteromeric potassium channels in those cells. Figure 5E is another field of stomach cells obtained using probes specific for Isk2 and KCNQ1; again, numerous yellow cells are seen.

DETAILED DESCRIPTION OF THE INVENTION

For the purposes of this invention:

“Substantially free from other proteins” means at least 90%, preferably 95%, more preferably 99%, and even more preferably 99.9%, free of other proteins.

5 Thus, a human IsK2 subunit protein preparation that is substantially free from other proteins will contain, as a percent of its total protein, no more than 10%, preferably no more than 5%, more preferably no more than 1%, and even more preferably no more than 0.1%, of proteins that are not human IsK2 subunit proteins. Whether a given human IsK2 subunit protein preparation is substantially free from other proteins can
10 be determined by conventional techniques of assessing protein purity such as, *e.g.*, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) combined with appropriate detection methods, *e.g.*, silver staining or immunoblotting.

“Substantially free from other nucleic acids” means at least 90%, preferably 95%, more preferably 99%, and even more preferably 99.9%, free of other
15 nucleic acids. Thus, a human IsK2 subunit DNA preparation that is substantially free from other nucleic acids will contain, as a percent of its total nucleic acid, no more than 10%, preferably no more than 5%, more preferably no more than 1%, and even more preferably no more than 0.1%, of nucleic acids that do not encode human IsK2. Whether a given human IsK2 subunit DNA preparation is substantially free from
20 other nucleic acids can be determined by conventional techniques of assessing nucleic acid purity such as, *e.g.*, agarose gel electrophoresis combined with appropriate staining methods, *e.g.*, ethidium bromide staining, or by sequencing.

A “conservative amino acid substitution” refers to the replacement of one amino acid residue by another, chemically similar, amino acid residue. Examples
25 of such conservative substitutions are: substitution of one hydrophobic residue (isoleucine, leucine, valine, or methionine) for another; substitution of one polar residue for another polar residue of the same charge (*e.g.*, arginine for lysine; glutamic acid for aspartic acid); substitution of one aromatic amino acid (tryptophan, tyrosine, or phenylalanine) for another.

30 A polypeptide has “substantially the same biological activity as the human IsK2 subunit protein” if that polypeptide is able to combine with another potassium channel subunit so as to form a complex that constitutes a functional potassium channel where the polypeptide confers upon the complex (as compared with the other subunit alone) altered electrophysiological or pharmacological

properties that are similar to the electrophysiological or pharmacological properties that the IsK2 protein confers on the other subunit and where the polypeptide has an amino acid sequence that is at least about 50% identical to SEQ.ID.NO.:2 when measured by such standard programs as BLAST or FASTA. Examples of other potassium channel subunits with which the polypeptide may combine are: KvQLT1, KvLQT2, KvLQT3, KvLQT4, KvLQT5, and h-erg.

The present invention relates to the identification and cloning of DNA encoding the human IsK2 protein. A cDNA was identified in a search of genomic DNA and EST databases as a clone encoding a protein with homology to the IsK protein. The IsK protein (also known as minK) is a subunit of two potassium channels underlying repolarizing cardiac K⁺ currents (I_{Ks} and I_{Kr}). In view of its homology to the IsK protein, the protein encoded by the cDNA was named IsK2. Northern analysis of the expression pattern of IsK2 demonstrated strong expression in human stomach and very weak expression in colon and small intestine, with no expression detected in any other tissue tested (Figure 4A). More detailed analysis, by *in situ* hybridization and immunohistochemistry, demonstrated that expression of this channel protein in human stomach is largely in the G cells (*i.e.*, gastrin secreting cells of the stomach; see Figure 3A-F) with very minor expression also in the gastric smooth muscle.

Chromosomal mapping studies have shown that the IsK2 gene maps to 21q22.1, on the same chromosome and within about 80 kb of the IsK gene, in the opposite orientation.

The present invention provides DNAs encoding the human IsK2 subunit that are substantially free from other nucleic acids. The present invention also provides isolated and/or recombinant DNA molecules encoding the human IsK2 subunit. The present invention provides DNA molecules substantially free from other nucleic acids comprising the nucleotide sequence shown in SEQ.ID.NO.:1.

The present invention includes isolated DNA molecules as well as DNA molecules that are substantially free from other nucleic acids comprising the coding region of SEQ.ID.NO.:1. Accordingly, the present invention includes isolated DNA molecules and DNA molecules substantially free from other nucleic acids having a sequence comprising positions 79-447 (the coding sequence) of SEQ.ID.NO.:1.

Also included are recombinant DNA molecules having a nucleotide sequence comprising positions 79-447 of SEQ.ID.NO.:1. The novel DNA sequences of the present invention encoding the human IsK2 subunit, in whole or in part, can be linked with other DNA sequences, *i.e.*, DNA sequences to which the human IsK2 subunit is not naturally linked, to form "recombinant DNA molecules" encoding the human IsK2 subunit. Such other sequences can include DNA sequences that control transcription or translation such as, *e.g.*, translation initiation sequences, internal ribosome entry sites, promoters for RNA polymerase II, transcription or translation termination sequences, enhancer sequences, sequences that control replication in microorganisms, sequences that confer antibiotic resistance, or sequences that encode a polypeptide "tag" such as, *e.g.*, a polyhistidine tract, the FLAG epitope, or the myc epitope. The novel DNA sequences of the present invention can be inserted into vectors such as plasmids, cosmids, viral vectors, P1 artificial chromosomes, or yeast artificial chromosomes.

Included in the present invention are DNA sequences that hybridize to SEQ.ID.NO.:1 under conditions of high stringency. By way of example, and not limitation, a procedure using conditions of high stringency is as follows: Prehybridization of filters containing DNA is carried out for 2 hr. to overnight at 65°C in buffer composed of 6X SSC, 5X Denhardt's solution, and 100 µg/ml denatured salmon sperm DNA. Filters are hybridized for 12 to 48 hrs at 65°C in prehybridization mixture containing 100 µg/ml denatured salmon sperm DNA and 5-20 X 10⁶ cpm of ³²P-labeled probe. Washing of filters is done at 37°C for 1 hr in a solution containing 2X SSC, 0.1% SDS. This is followed by a wash in 0.1X SSC, 0.1% SDS at 50°C for 45 min. before autoradiography.

Other procedures using conditions of high stringency would include either a hybridization carried out in 5XSSC, 5X Denhardt's solution, 50% formamide at 42°C for 12 to 48 hours or a washing step carried out in 0.2X SSPE, 0.2% SDS at 65°C for 30 to 60 minutes.

Reagents mentioned in the foregoing procedures for carrying out high stringency hybridization are well known in the art. Details of the composition of these reagents can be found in, *e.g.*, Sambrook, Fritsch, and Maniatis, 1989, Molecular Cloning: A Laboratory Manual, second edition, Cold Spring Harbor Laboratory Press. In addition to the foregoing, other conditions of high stringency which may be used are well known in the art.

The degeneracy of the genetic code is such that, for all but two amino acids, more than a single codon encodes a particular amino acid. This allows for the construction of synthetic DNA that encodes the human IsK2 subunit protein where the nucleotide sequence of the synthetic DNA differs significantly from the nucleotide sequences of SEQ.ID.NO:1, but still encodes the same human IsK2 subunit protein as SEQ.ID.NO:1. Such synthetic DNAs are intended to be within the scope of the present invention.

Mutated forms of SEQ.ID.NO:1 are intended to be within the scope of the present invention. In particular, mutated forms of SEQ.ID.NO:1 encoding a protein that, when combined with other potassium channel subunits, give rise to potassium channels having altered voltage sensitivity, current carrying properties, pharmacologic properties, or other properties as compared to potassium channels formed by combination of wild-type Isk2 protein with the other potassium channel subunit, are within the scope of the present invention. Such mutant forms can differ from SEQ.ID.NO:1 by having nucleotide deletions, substitutions, or additions.

Also intended to be within the scope of the present invention are RNA molecules having sequences corresponding to SEQ.ID.NO:1. Antisense oligonucleotides, DNA or RNA, that are the reverse complements of SEQ.ID.NO:1, or portions thereof, are also within the scope of the present invention. In addition, polynucleotides based on SEQ.ID.NO:1 in which a small number of positions are substituted with non-natural or modified nucleotides such as inosine, methyl-cytosine, or deaza-guanosine are intended to be within the scope of the present invention. Polynucleotides of the present invention can also include sequences based on SEQ.ID.NO:1 but in which non-natural linkages between the nucleotides are present. Such non-natural linkages can be, *e.g.*, methylphosphonates, phosphorothioates, phosphorodithionates, phosphoroamidites, and phosphate esters. Polynucleotides of the present invention can also include sequences based on SEQ.ID.NO:1 but having de-phospho linkages as bridges between nucleotides, *e.g.*, siloxane, carbonate, carboxymethyl ester, acetamidate, carbamate, and thioether bridges. Other internucleotide linkages that can be present include N-vinyl, methacryloxyethyl, methacrylamide, or ethyleneimine linkages. Peptide nucleic acids based upon SEQ.ID.NO:1 are also included in the present invention.

Another aspect of the present invention includes host cells that have been engineered to contain and/or express DNA sequences encoding the human IsK2

subunit protein. Such recombinant host cells can be cultured under suitable conditions to produce human IsK2 subunit protein. An expression vector containing DNA encoding the human IsK2 subunit protein can be used for the expression of the human IsK2 subunit protein in a recombinant host cell. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to, bacteria such as *E. coli*, fungal cells such as yeast, mammalian cells including, but not limited to, cell lines of human, bovine, porcine, monkey and rodent origin, amphibian cells such as *Xenopus* oocytes, and insect cells including but not limited to *Drosophila* and silkworm derived cell lines. Cells and cell lines which are suitable for recombinant expression of the human IsK2 subunit protein and which are widely available, include but are not limited to, L cells L-M(TK⁻) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), CPAE (ATCC CCL 209), Saos-2 (ATCC HTB-85), ARPE-19 human retinal pigment epithelium (ATCC CRL-2302), *Xenopus* melanophores, and *Xenopus* oocytes.

A variety of mammalian expression vectors can be used to express recombinant human IsK2 subunit protein in mammalian cells. Commercially available mammalian expression vectors which are suitable include, but are not limited to, pMC1neo (Stratagene), pSG5 (Stratagene), pcDNAI and pcDNAIamp, pcDNA3, pcDNA3.1, pCR3.1 (Invitrogen), EBO-pSV2-neo (ATCC 37593), pBPV-1(8-2) (ATCC 37110), pdBPV-MMTneo(342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), pIZD35 (ATCC 37565), and pSV2-dhfr (ATCC 37146). Another suitable vector is the PT7TS oocyte expression vector.

Following expression in recombinant cells, human IsK2 subunit protein can be purified by conventional techniques to a level that is substantially free from other proteins. Techniques that can be used include ammonium sulfate precipitation, hydrophobic or hydrophilic interaction chromatography, ion exchange chromatography, affinity chromatography, phosphocellulose chromatography, size exclusion chromatography, preparative gel electrophoresis, and alcohol precipitation. In some cases, it may be advantageous to employ protein denaturing and/or refolding steps in addition to such techniques.

Certain voltage-gated potassium channel subunits have been found to require the expression of other voltage-gated potassium channel subunits in order to be properly expressed at high levels and inserted in membranes. For example, co-expression of KCNQ3 appears to enhance the expression of KCNQ2 in *Xenopus* oocytes (Wang et al., 1998, Science 282:1890-1893). Also, some voltage-gated potassium channel Kv1 α subunits require other related α subunits or Kv β 2 subunits (Shi et al., 1995, Neuron 16:843-852). Accordingly, the recombinant expression of the human IsK2 subunit proteins may under certain circumstances benefit from the co-expression of other potassium channel proteins and such co-expression is intended to be within the scope of the present invention. A particularly preferred form of co-expression is the co-expression of a human IsK2 subunit protein with a human KvQLT1 subunit protein or with a human h-erg subunit protein. Such co-expression can be effected by transfecting an expression vector encoding a human IsK2 subunit protein into a cell that naturally expresses a human KvQLT1 subunit protein or a human h-erg subunit protein. Alternatively, an expression vector encoding a human IsK2 subunit protein can be transfected into a cell in which an expression vector encoding a human KvQLT1 subunit protein or a human h-erg subunit protein has also been transfected. Preferably, such a cell does not naturally express human KvQLT1 subunit protein or human h-erg subunit protein.

The present invention includes human IsK2 subunit proteins substantially free from other proteins. The amino acid sequence of the full-length human IsK2 subunit protein is shown in SEQ.ID.NO.:2. Thus, the present invention includes human IsK2 subunit protein substantially free from other proteins having the amino acid sequence SEQ.ID.NO.:2. The present invention also includes isolated human IsK2 subunit protein having the amino acid sequence SEQ.ID.NO.:2.

Mutated forms of human IsK2 subunit protein are intended to be within the scope of the present invention. In particular, mutated forms of SEQ.ID.NO.:2 that give rise to potassium channels having altered electrophysiological or pharmacological properties when combined with other potassium channel subunits are within the scope of the present invention.

As with many proteins, it is possible to modify many of the amino acids of the human IsK2 subunit protein and still retain substantially the same biological activity as for the original protein. Thus, the present invention includes modified human IsK2 subunit proteins which have amino acid deletions, additions, or

substitutions but that still retain substantially the same biological activity as naturally occurring human IsK2 subunit protein. It is generally accepted that single amino acid substitutions do not usually alter the biological activity of a protein (see, *e.g.*, Molecular Biology of the Gene, Watson *et al.*, 1987, Fourth Ed., The Benjamin/Cummings Publishing Co., Inc., page 226; and Cunningham & Wells, 1989, Science 244:1081-1085). Accordingly, the present invention includes polypeptides where one amino acid substitution has been made in SEQ.ID.NO:2 wherein the polypeptides still retain substantially the same biological activity as naturally occurring human IsK2 subunit protein. The present invention also includes polypeptides where two or more amino acid substitutions have been made in SEQ.ID.NO:2 wherein the polypeptides still retain substantially the same biological activity as naturally occurring human IsK2 subunit protein. In particular, the present invention includes embodiments where the above-described substitutions are conservative substitutions. In particular, the present invention includes embodiments where the above-described substitutions do not occur in conserved positions. Conserved positions are those positions in which the human IsK2 and human minK proteins have the same amino acid (see Figure 2).

The human IsK2 subunit proteins of the present invention may contain post-translational modifications, *e.g.*, covalently linked carbohydrate, phosphorylation, myristoylation, palmytoylation, *etc.*

The present invention also includes chimeric human IsK2 subunit proteins. Chimeric human IsK2 subunit proteins consist of a contiguous polypeptide sequence of at least a portion of a human IsK2 subunit protein fused to a polypeptide sequence that is not from a human IsK2 subunit protein.

The present invention also includes isolated human IsK2 subunit protein and DNA encoding the isolated subunit. Use of the term "isolated" indicates that the human IsK2 subunit protein or DNA has been removed from its normal cellular environment. Thus, an isolated human IsK2 subunit protein may be in a cell-free solution or placed in a different cellular environment from that in which it occurs naturally. The term isolated does not imply that an isolated human IsK2 subunit protein is the only protein present, but instead means that the isolated human IsK2 subunit protein is at least 95% free of non-amino acid material (*e.g.*, nucleic acids, lipids, carbohydrates) naturally associated with the human IsK2 subunit protein. Thus, a human IsK2 subunit protein that is expressed in bacteria or even in eukaryotic

cells which do not naturally (*i.e.*, without human intervention) express it through recombinant means is an "isolated human IsK2 subunit protein."

It is known that certain potassium channels subunits can interact to form heteromeric complexes resulting in functional potassium channels. For example, KCNQ2 and KCNQ3 can assemble to form a heteromeric functional potassium channel (Wang et al., 1998, Science 282:1890-1893). Accordingly, it is believed likely that the human IsK2 subunit protein of the present invention will also be able to form heteromeric structures with other proteins where such heteromeric structures constitute functional potassium channels. Thus, the present invention includes such heteromers comprising human IsK2 subunit protein. Preferred heteromers are those in which the human IsK2 subunit proteins of the present invention forms heteromers with human KvQLT1 or h-erg.

DNA encoding the human IsK2 subunit protein can be obtained by methods well known in the art. For example, a cDNA fragment encoding full-length human IsK2 protein can be isolated from a human stomach cDNA library by using the polymerase chain reaction (PCR) employing suitable primer pairs. Such primer pairs can be selected based upon the DNA sequence encoding the human IsK2 protein shown in Figure 1A as SEQ.ID.NO.:1. Suitable primer pairs would be, *e.g.*:

5'- ATA GCC AAA TCC AGA AAA -3' (SEQ.ID.NO.:4) and
5'-GCT TGG TGC CTT TCT CCC-3' (SEQ.ID.NO.:5).

The above primers are meant to be illustrative only; one skilled in the art would readily be able to design other suitable primers based upon SEQ.ID.NO.:1. Such primers could be produced by methods of oligonucleotide synthesis that are well known in the art.

PCR reactions can be carried out with a variety of thermostable enzymes including but not limited to AmpliTaq, AmpliTaq Gold, or Vent polymerase. For AmpliTaq, reactions can be carried out in 10 mM Tris-Cl, pH 8.3, 2.0 mM MgCl₂, 200 μ M of each dNTP, 50 mM KCl, 0.2 μ M of each primer, 10 ng of DNA template, 0.05 units/ μ l of AmpliTaq. The reactions are heated at 95°C for 3 minutes and then cycled 35 times using suitable cycling parameters, including, but not limited to, 95°C, 20 seconds, 62°C, 20 seconds, 72°C, 3 minutes. In addition to these conditions, a variety of suitable PCR protocols can be found in PCR Primer, A

Laboratory Manual, edited by C.W. Dieffenbach and G.S. Dveksler, 1995, Cold Spring Harbor Laboratory Press; or PCR Protocols: A Guide to Methods and Applications, Michael *et al.*, eds., 1990, Academic Press .

5 Since the IsK2 channel subunit of the present invention is homologous to other potassium channel subunits (see Figure 2), it is desirable to sequence the clones obtained by the herein-described methods, in order to verify that the desired IsK2 subunit has in fact been obtained.

10 By these methods, cDNA clones encoding the human IsK2 subunit protein can be obtained. These cDNA clones can be subcloned into suitable cloning vectors or expression vectors, *e.g.*, the mammalian expression vector pcDNA3.1 (Invitrogen, San Diego, CA). Human IsK2 subunit protein can then be produced by transferring expression vectors encoding the subunit or portions thereof into suitable host cells and growing the host cells under appropriate conditions. Human IsK2 subunit protein can then be isolated by methods well known in the art.

15 As an alternative to the above-described PCR methods, cDNA clones encoding the human IsK2 subunit protein can be isolated from cDNA libraries using as a probe oligonucleotides specific for the human IsK2 subunit and methods well known in the art for screening cDNA libraries with oligonucleotide probes. Such methods are described in, *e.g.*, Sambrook *et al.*, 1989, *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, *DNA Cloning: A Practical Approach*, MRL Press, Ltd., Oxford, U.K., Vol. I, II. Oligonucleotides that are specific for human IsK2 subunit protein and that can be used to screen cDNA libraries can be readily designed based upon the DNA sequence shown in Figure 1A and can be synthesized by methods
20 well-known in the art.
25

30 Genomic clones containing the human IsK2 subunit gene can be obtained by PCR using human genomic DNA as the template or from commercially available human PAC or BAC libraries available from Research Genetics, Huntsville, AL. Alternatively, one may prepare genomic libraries, *e.g.*, in P1 artificial chromosome vectors, from which genomic clones containing the human IsK2 subunit gene can be isolated, using probes based upon the the human IsK2 subunit DNA sequence disclosed herein. Methods of preparing such libraries are known in the art (see, *e.g.*, Ioannou *et al.*, 1994, *Nature Genet.* 6:84-89).

The novel DNA sequences of the present invention can be used in various diagnostic methods. The present invention provides diagnostic methods for determining whether a patient carries a mutation in the human IsK2 subunit gene. In broad terms, such methods comprise determining the DNA sequence of a region in or
5 near the human IsK2 subunit gene from the patient and comparing that sequence to the sequence from the corresponding region of the human IsK2 subunit gene from a non-affected person, *i.e.*, a person who does not have the condition which is being diagnosed, where a difference in sequence between the DNA sequence of the gene from the patient and the DNA sequence of the gene from the non-affected person
10 indicates that the patient has a mutation in the human IsK2 subunit gene.

The present invention also provides oligonucleotide probes, based upon SEQ.ID.NO:1 that can be used in diagnostic methods to identify patients having mutated forms of the human IsK2 subunit, to determine the level of expression of RNA encoding the human IsK2 subunit, or to isolate genes homologous to the human
15 IsK2 subunit from other species. In particular, the present invention includes DNA oligonucleotides comprising at least about 10, 15, or 18 contiguous nucleotides of SEQ.ID.NO:1 where the oligonucleotide probe comprises no stretch of contiguous nucleotides longer than 5 from SEQ.ID.NO:1 other than the said at least about 10, 15, or 18 contiguous nucleotides. The oligonucleotides can be substantially free from
20 other nucleic acids. Also provided by the present invention are corresponding RNA oligonucleotides. The DNA or RNA oligonucleotides can be packaged in kits.

The present invention makes possible the recombinant expression of human IsK2 subunit protein in various cell types. Such recombinant expression makes possible the study of this protein so that its biochemical activity and its role in
25 various diseases such as gastric motility disorders or disorders of gastric acid secretion can be elucidated.

The present invention also makes possible the development of assays which measure the biological activity of potassium channels containing human IsK2 subunit protein. Such assays using recombinantly expressed human IsK2 subunit
30 protein are especially of interest. Such assays can be used to screen libraries of compounds or other sources of compounds to identify compounds that are activators or inhibitors of the activity of potassium channels containing human IsK2 subunit protein. Such identified compounds can serve as "leads" for the development of

pharmaceuticals that can be used to treat patients having diseases in which it is beneficial to enhance or suppress potassium channel activity.

In versions of the above-described assays, potassium channels containing mutant human IsK2 subunit proteins are used and inhibitors or activators of the activity of the mutant potassium channels are identified.

Preferred cell lines for recombinant expression of human IsK2 subunit protein are those which do not express endogenous potassium channels (*e.g.*, CV-1, NIH-3T3, CHO-K1, COS-7). Such cell lines can be loaded with ^{86}Rb , an ion which can pass through potassium channels. The ^{86}Rb -loaded cells can be exposed to collections of substances (*e.g.*, combinatorial libraries, natural products, analogues of lead compounds produced by medicinal chemistry) and those substances that are able to alter ^{86}Rb efflux identified. Such substances are likely to be activators or inhibitors of potassium channels containing human IsK2 subunit protein.

Activators and inhibitors of potassium channels containing human IsK2 subunit protein are likely to be substances that are capable of binding to potassium channels containing human IsK2 subunit protein. Thus, one type of assay determines whether one or more of a collection of substances is capable of such binding.

Accordingly, the present invention provides a method for identifying substances that bind to potassium channels containing human IsK2 subunit protein comprising:

- (a) providing cells expressing a potassium channel containing human IsK2 subunit protein;
- (b) exposing the cells to a substance that is not known to bind potassium channels containing human IsK2 subunit protein;
- (c) determining the amount of binding of the substance to the cells;
- (d) comparing the amount of binding in step (c) to the amount of binding of the substance to control cells where the control cells are substantially identical to the cells of step (a) except that the control cells do not express human IsK2 subunit protein;

where if the amount of binding in step (c) is greater than the amount of binding of the substance to control cells, then the substance binds to potassium channels containing human IsK2 subunit protein.

An example of control cells that are substantially identical to the cells of step (a) would be a parent cell line where the parent cell line is transfected with an expression vector encoding IsK2 protein in order to produce the cells expressing a potassium channel containing human IsK2 protein of step (a).

5 Another version of this assay makes use of compounds that are known to bind to potassium channels containing human IsK2 subunit protein. New binders are identified by virtue of their ability to enhance or block the binding of these known compounds. Substances that have this ability are likely themselves to be inhibitors or activators of potassium channels containing human IsK2 subunit protein.

10 Accordingly, the present invention includes a method of identifying substances that bind potassium channels containing human IsK2 subunit protein and thus are likely to be inhibitors or activators of potassium channels containing human IsK2 subunit protein comprising:

- 15 (a) providing cells expressing potassium channels containing human IsK2 subunit protein;
- (b) exposing the cells to a compound that is known to bind to the potassium channels containing human IsK2 subunit protein in the presence and in the absence of a substance not known to bind to potassium channels containing human IsK2 subunit protein;
- 20 (c) determining the amount of binding of the compound to the cells in the presence and in the absence of a substance not known to bind to potassium channels containing human IsK2 subunit protein;

where if the amount of binding of the compound in the presence of the substance differs from the amount of binding in the absence of the substance, then the
25 substance binds potassium channels containing human IsK2 subunit protein and is likely to be an inhibitor or activator of potassium channels containing human IsK2 subunit protein.

Generally, the known compound is labeled (*e.g.*, radioactively, enzymatically, fluorescently) in order to facilitate measuring its binding to the
30 potassium channels.

Once a substance has been identified by the above-described methods, it can be assayed in functional tests, such as those described herein, in order to determine whether it is an inhibitor or an activator.

In particular embodiments, the compound known to bind potassium channels containing human IsK2 subunit protein is selected from the group consisting of: methanesulfonanilide class III antiarrhythmics such as E-4031 (Abbott et al., 1999, Cell 97:175-187), derivatives of E-4031 (e.g., MK-499 (Lynch et al., 1994, J.

- 5 Pharmacol. Exp. Therapeut. 269:541-554)), and compounds that block or activate IKs (e.g., L-364,373 (Salata et al., 1998, Mol. Pharmacol. 53:220-230), L-768,673 (Selnick et al., 1997, J. Med. Chem. 40:3865-3868), or L-735,821 (Salata et al., 1996, Circulation 94:I-529)).

- 10 The present invention includes a method of identifying activators or inhibitors of potassium channels containing human IsK2 subunit protein comprising:

- (a) recombinantly expressing human IsK2 subunit protein or mutant human IsK2 subunit protein in a host cell so that the recombinantly expressed human IsK2 subunit protein forms potassium channels either by itself or by forming heteromers with other potassium channel subunit proteins;
- 15 (b) measuring the biological activity of the potassium channels formed in step (a) in the presence and in the absence of a substance suspected of being an activator or an inhibitor of potassium channels containing human IsK2 subunit protein;

- where a change in the biological activity of the potassium channels formed in step (a) in the presence as compared to the absence of the substance indicates that the substance is an activator or an inhibitor of potassium channels containing human IsK2 subunit protein.
- 20

- It may be advantageous to recombinantly express the other subunits of potassium channels such as, e.g., the human KvQLT1 or h-erg subunit. Alternatively,
- 25 it may be advantageous to use host cells that endogenously express such other subunits.

In particular embodiments, the biological activity is the production of a voltage-gated potassium current or the efflux of ^{86}Rb .

- In particular embodiments, a vector encoding human IsK2 subunit protein is transferred into *Xenopus* oocytes in order to cause the expression of human IsK2 subunit protein in the oocytes. Alternatively, RNA encoding human IsK2 subunit protein can be prepared *in vitro* and injected into the oocytes, also resulting in the expression of human IsK2 subunit protein in the oocytes. Following expression of the human IsK2 subunit protein in the oocytes, and following the formation of
- 30

potassium channels containing these subunits and other potassium subunits (which other subunits may also be transferred into the oocytes), membrane currents are measured after the transmembrane voltage is changed in steps. A change in membrane current is observed when the potassium channels open, allowing potassium ion flow. Similar oocyte studies were reported for KCNQ2 and KCNQ3 potassium channels in Wang et al., 1998, Science 282:1890-1893 and for minK-containing channels by Goldstein & Miller, 1991, Neuron 7:403-408. These references and references cited therein can be consulted for guidance as to how to carry out such studies.

Inhibitors of potassium channels containing human IsK2 subunit protein can be identified by exposing the oocytes to substances or collections of substances and determining whether the substances can block or diminish the membrane currents observed in the absence of the substance.

Accordingly, the present invention provides a method of identifying inhibitors of potassium channels containing human IsK2 subunit protein comprising:

- (a) expressing human IsK2 subunit protein in *Xenopus* oocytes such that potassium channels containing the human IsK2 subunit protein are formed;
- (b) changing the transmembrane potential of the oocytes in the presence and the absence of a substance suspected of being an inhibitor of potassium channels containing human IsK2 subunit protein;
- (c) measuring membrane potassium currents following step (b); where if the potassium membrane currents measured in step (c) are greater in the absence rather than in the presence of the substance, then the substance is an inhibitor of potassium channels containing human IsK2 subunit protein.

The present invention also includes assays for the identification of activators and inhibitors of potassium channels containing human IsK2 subunit protein that are based upon fluorescence resonance energy transfer (FRET) between a first and a second fluorescent dye where the first dye is bound to one side of the plasma membrane of a cell expressing potassium channels containing human IsK2 subunit protein and the second dye is free to shuttle from one face of the membrane to the other face in response to changes in membrane potential. In certain embodiments, the first dye is impenetrable to the plasma membrane of the cells and is bound predominately to the extracellular surface of the plasma membrane. The second dye is trapped within the plasma membrane but is free to diffuse within the membrane.

At normal (*i.e.*, negative) resting potentials of the membrane, the second dye is bound predominately to the inner surface of the extracellular face of the plasma membrane, thus placing the second dye in close proximity to the first dye. This close proximity allows for the generation of a large amount of FRET between the two dyes.

- 5 Following membrane depolarization, the second dye moves from the extracellular face of the membrane to the intracellular face, thus increasing the distance between the dyes. This increased distance results in a decrease in FRET, with a corresponding increase in fluorescent emission derived from the first dye and a corresponding decrease in the fluorescent emission from the second dye. In this way, the amount of
- 10 FRET between the two dyes can be used to measure the polarization state of the membrane. For a description of this technique, see González & Tsien, 1997, Chemistry & Biology 4:269-277. See also González & Tsien, 1995, Biophys. J. 69:1272-1280 and U.S. Patent No. 5,661,035.

- In certain embodiments, the first dye is a fluorescent lectin or a
- 15 fluorescent phospholipid that acts as the fluorescent donor. Examples of such a first dye are: a coumarin-labeled phosphatidylethanolamine (*e.g.*, N-(6-chloro-7-hydroxy-2-oxo-2H--1-benzopyran-3-carboxamidoacetyl)-dimyristoylphosphatidyl-ethanolamine) or N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-dipalmitoylphosphatidylethanolamine); a fluorescently-labeled lectin (*e.g.*,
- 20 fluorescein-labeled wheat germ agglutinin). In certain embodiments, the second dye is an oxonol that acts as the fluorescent acceptor. Examples of such a second dye are: bis(1,3-dialkyl-2-thiobarbiturate)trimethineoxonols (*e.g.*, bis(1,3-dihexyl-2-thiobarbiturate)trimethineoxonol) or pentamethineoxonol analogues (*e.g.*, bis(1,3-dihexyl-2-thiobarbiturate)pentamethineoxonol; or bis(1,3-dibutyl-2-
- 25 thiobarbiturate)pentamethineoxonol). See González & Tsien, 1997, Chemistry & Biology 4:269-277 for methods of synthesizing various dyes suitable for use in the present invention. In certain embodiments, the assay may comprise a natural carotenoid, *e.g.*, astaxanthin, in order to reduce photodynamic damage due to singlet oxygen.

- 30 The above described assays can be utilized to discover activators and inhibitors of potassium channels containing human Isk2 subunit protein. Such assays will generally utilize cells that express potassium channels containing human Isk2 subunit protein, *e.g.*, by transfection with expression vectors encoding human Isk2 subunit protein and, optionally, other potassium channel subunits. In such cells,

increases in membrane potential (*i.e.*, depolarizations) may open the potassium channels. This will result in potassium efflux, tending to counteract the depolarization. In other words, the cells will tend to repolarize. The presence of an inhibitor of the potassium channel containing IsK2 protein will prevent, or diminish, this repolarization. Thus, membrane potential will tend to become more positive in the presence of inhibitors. Agonists of the potassium channel will open this channel and thus tend to hyperpolarize the membrane potential. Changes in membrane potential (depolarizations and hyperpolarizations) that are caused by inhibitors and agonists of potassium channels containing IsK2 protein can be monitored by the assays using FRET described above.

Accordingly, the present invention provides a method of identifying activators of potassium channels containing human IsK2 subunit protein comprising:

- (a) providing test cells comprising:
 - (1) an expression vector that directs the expression of human IsK2 subunit protein in the cells so that potassium channels containing human IsK2 subunit protein are formed in the cells;
 - (2) a first fluorescent dye, where the first dye is bound to one side of the plasma membrane of the cells; and
 - (3) a second fluorescent dye, where the second fluorescent dye is free to shuttle from one face of the plasma membrane of the cells to the other face in response to changes in membrane potential;
- (b) exposing the test cells to a substance that is suspected of being an activator of potassium channels containing human IsK2 subunit protein;
- (c) measuring the amount of fluorescence resonance energy transfer (FRET) in the test cells that have been exposed to the substance;
- (d) comparing the amount of FRET exhibited by the test cells that have been exposed to the substance with the amount of FRET exhibited by control cells;

where if the amount of FRET exhibited by the test cells is greater than the amount of FRET exhibited by the control cells, the substance is an activator of potassium channels containing human IsK2 subunit protein;

where the control cells are either (1) cells that are essentially the same as the test cells except that they do not comprise at least one of the items listed at (a)

(1)-(3) but have been exposed to the substance; or (2) test cells that have not been exposed to the substance.

The present invention also provides a method of identifying inhibitors of potassium channels containing human Isk2 subunit protein comprising:

- 5 (a) providing test cells comprising:
 - (1) an expression vector that directs the expression of human Isk2 subunit protein in the cells so that potassium channels containing human Isk2 subunit proteins are formed in the cells;
 - (2) a first fluorescent dye, where the first dye is bound to
10 one side of the plasma membrane of the cells; and
 - (3) a second fluorescent dye, where the second fluorescent dye is free to shuttle from one face of the plasma membrane of the cells to the other face in response to changes in membrane potential;
- (b) exposing the test cells to a substance that is suspected of being
15 an inhibitor of potassium channels containing human Isk2 subunit protein;
- (c) measuring the amount of fluorescence resonance energy transfer (FRET) in the test cells that have been exposed to the substance;
- (d) comparing the amount of FRET exhibited by the test cells that have been exposed to the substance with the amount of FRET exhibited by control
20 cells;

where if the amount of FRET exhibited by the test cells is less than the amount of FRET exhibited by the control cells, the substance is an inhibitor of potassium channels containing human Isk2 subunit protein;

- 25 where the control cells are either (1) cells that are essentially the same as the test cells except that they do not comprise at least one of the items listed at (a) (1)-(3) but have been exposed to the substance; or (2) test cells that have not been exposed to the substance.

In a variation of the assays described above, instead of the cell's membrane potential being allowed to reach steady state on its own, the membrane potential is artificially set at a potential in which the potassium channels containing human Isk2 subunit protein are open. This can be done, *e.g.*, by variation of the
30 external K⁺ concentration in a known manner (*e.g.*, increased concentrations of external K⁺). If such cells, having open potassium channels containing human Isk2 subunit protein, are exposed to inhibitors, the potassium channels will close, and the

cells' membrane potentials will be depolarized. This depolarization can be observed as a decrease in FRET.

Accordingly, the present invention provides a method of identifying inhibitors of potassium channels containing human IsK2 subunit protein comprising:

5 (a) providing cells comprising:

(1) an expression vector that directs the expression of human IsK2 subunit protein in the cells so that potassium channels containing human IsK2 subunit protein are formed in the cells;

10 (2) a first fluorescent dye, where the first dye is bound to one side of the plasma membrane of the cells; and

(3) a second fluorescent dye, where the second fluorescent dye is free to shuttle from one face of the plasma membrane of the cells to the other face in response to changes in membrane potential;

15 (b) adjusting the membrane potential of the cells such that the ion channel formed by the potassium channels containing human IsK2 subunit protein is open;

(c) measuring the amount of fluorescence resonance energy transfer (FRET) in the test cells;

20 (d) repeating step (b) and step (c) while the cells are exposed to a substance that is suspected of being an inhibitor of potassium channels containing human IsK2 subunit protein;

25 where if the amount of FRET exhibited by the cells that are exposed to the substance is less than the amount of FRET exhibited by the cells that have not been exposed to the substance, then the substance is an inhibitor of potassium channels containing human IsK2 subunit protein.

In particular embodiments of the above-described methods, the expression vector is transfected into the test cells.

30 In particular embodiments of the above-described methods, the human IsK2 subunit protein has the amino acid sequence shown in:SEQ.ID.NO.:2. In particular embodiments of the above-described methods, the expression vector comprises positions 79-447 of SEQ.ID.NO.:1.

In particular embodiments of the above-described methods, the first fluorescent dye is selected from the group consisting of: a fluorescent lectin; a fluorescent phospholipid; a coumarin-labeled phosphatidylethanolamine; N-(6-chloro-

7-hydroxy-2-oxo-2H--1-benzopyran-3-carboxamidoacetyl)-dimyristoylphosphatidyl-ethanolamine); N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-dipalmitoylphosphatidylethanolamine); and fluorescein-labeled wheat germ agglutinin.

5 In particular embodiments of the above-described methods, the second fluorescent dye is selected from the group consisting of: an oxonol that acts as the fluorescent acceptor; bis(1,3-dialkyl-2-thiobarbiturate)trimethineoxonols; bis(1,3-dihexyl-2-thiobarbiturate)trimethineoxonol; bis(1,3-dialkyl-2-thiobarbiturate)quatramethineoxonols; bis(1,3-dialkyl-2-thiobarbiturate)pentamethineoxonols; 10 bis(1,3-dihexyl-2-thiobarbiturate)pentamethineoxonol; bis(1,3-dibutyl-2-thiobarbiturate)pentamethineoxonol; and bis(1,3-dialkyl-2-thiobarbiturate)hexamethineoxonols.

In a particular embodiments of the above-described methods, the cells are eukaryotic cells. In another embodiment, the cells are mammalian cells. In other 15 embodiments, the cells are L cells L-M(TK⁻) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C1271 (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), *Xenopus* 20 melanophores, or *Xenopus* oocytes.

In particular embodiments of the above-described methods, the control cells do not comprise item (a)(1) but do comprise items (a)(2) and (a)(3).

In assays to identify activators or inhibitors of potassium channels containing human Isk2 subunit protein, it may be advantageous to co-express another 25 potassium channel subunit besides the human Isk2 subunit. In particular, it may be advantageous to co-express a potassium channel subunit such as human KvQLT1 (Sanguinetti et al., 1996, Nature 384:80-83; GenBank accession nos. U71077 and U40990), *Xenopus* KvQLT1 (Sanguinetti et al., 1996, Nature 384:80-83; GenBank accession no. U71076) or h-erg (Warmke & Ganetzky, 1994, Proc. Natl. Acad. Sci. 30 USA 91:3438-3442; GenBank accession no. U04270) . Preferably, this is done by co-transfecting into the cells an expression vector encoding the other subunit.

While the above-described methods are explicitly directed to testing whether "a" substance is an activator or inhibitor of potassium channels containing human Isk2 subunit protein, it will be clear to one skilled in the art that such methods

can be adapted to test collections of substances, *e.g.*, combinatorial libraries, phage display libraries, collections of natural products, to determine whether any members of such collections are activators or inhibitors of potassium channels containing human IsK2 subunit protein. Accordingly, the use of collections of substances, or individual members of such collections, as the substance in the above-described methods is within the scope of the present invention. In particular, it is envisioned that libraries that have been designed to incorporate chemical structures that are known to be associated with potassium ion channel modulation, *e.g.*, dihydrobenzopyran libraries for potassium channel activators (International Patent Publication WO 95/30642) or biphenyl-derivative libraries for potassium channel inhibitors (International Patent Publication WO 95/04277), will be of especial interest.

The present invention includes pharmaceutical compositions comprising activators or inhibitors of potassium channels comprising human IsK2 subunit protein that have been identified by the herein-described methods. The activators or inhibitors are generally combined with pharmaceutically acceptable carriers to form pharmaceutical compositions. Examples of such carriers and methods of formulation of pharmaceutical compositions containing activators or inhibitors and carriers can be found in Remington's Pharmaceutical Sciences, 18th Edition, 1990, Mack Publishing Co., Easton, PA. To form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain a therapeutically effective amount of the activators or inhibitors.

Therapeutic or prophylactic compositions are administered to an individual in amounts sufficient to treat or prevent conditions where the activity of potassium channels containing human IsK2 subunit protein is abnormal. The effective amount can vary according to a variety of factors such as the individual's condition, weight, gender, and age. Other factors include the mode of administration. The appropriate amount can be determined by a skilled physician. Generally, an effective amount will be from about 0.01 to about 1,000, preferably from about 0.1 to about 250, and even more preferably from about 1 to about 50 mg per adult human per day.

Compositions can be used alone at appropriate dosages. Alternatively, co-administration or sequential administration of other agents can be desirable.

The compositions can be administered in a wide variety of therapeutic dosage forms in conventional vehicles for administration. For example, the compositions can be administered in such oral dosage forms as tablets, capsules (each including timed release and sustained release formulations), pills, powders, granules, 5 elixirs, tinctures, solutions, suspensions, syrups and emulsions, or by injection. Likewise, they can also be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous, topical with or without occlusion, or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts.

10 Compositions can be administered in a single daily dose, or the total daily dosage can be administered in divided doses of two, three, four or more times daily. Furthermore, compositions can be administered in intranasal form via topical use of suitable intranasal vehicles, or via transdermal routes, using those forms of transdermal skin patches well known to those of ordinary skill in that art. To be administered in the form of a transdermal delivery system, the dosage administration 15 will, of course, be continuous rather than intermittent throughout the dosage regimen.

The dosage regimen utilizing the compositions is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal, hepatic and cardiovascular function of the patient; and 20 the particular composition thereof employed. A physician of ordinary skill can readily determine and prescribe the effective amount of the composition required to prevent, counter or arrest the progress of the condition. Optimal precision in achieving concentrations of composition within the range that yields efficacy without toxicity requires a regimen based on the kinetics of the composition's availability to 25 target sites. This involves a consideration of the distribution, equilibrium, and elimination of a composition.

The inhibitors and activators of potassium channels containing human IsK2 subunit protein will be useful for treating a variety of diseases involving excessive or insufficient potassium channel activity.

30 The inhibitors of the present invention are expected to be useful in conditions where currently marketed inhibitors of potassium channels have efficacy. For example, potassium channel antagonists are used as Class III antiarrhythmic agents to treat myocardial infarction. It is expected that the inhibitors of the present invention are likely to also be useful in this manner.

Human IsK2 protein is specifically expressed in the G cells of the stomach. G cells of the stomach secrete gastrin, which is involved in the regulation of acid secretion by parietal cells. Therefore, modulators of the activity of potassium channels comprising IsK2 are likely to be useful in regulating gastrin secretion and acid production.

The IsK2 subunit of the present invention is useful in conjunction with screens designed to identify activators and inhibitors of other ion channels. When screening compounds in order to identify potential pharmaceuticals that specifically interact with a target ion channel, it is necessary to ensure that the compounds identified are as specific as possible for the target ion channel. To do this, it is necessary to screen the compounds against as wide an array as possible of ion channels that are similar to the target ion channel. Thus, in order to find compounds that are potential pharmaceuticals that interact with ion channel A, it is not enough to ensure that the compounds interact with ion channel A (the "plus target") and produce the desired pharmacological effect through ion channel A. It is also necessary to determine that the compounds do not interact with ion channels B, C, D, *etc.* (the "minus targets"). In general, as part of a screening program, it is important to have as many minus targets as possible (see Hodgson, 1992, *Bio/Technology* 10:973-980, at 980). Human IsK2 subunit protein, DNA encoding human IsK2 subunit protein, and recombinant cells that have been engineered to express human IsK2 subunit protein have utility in that they can be used as "minus targets" in screens designed to identify compounds that specifically interact with other ion channels. For example, Wang et al., 1998, *Science* 282:1890-1893 have shown that KCNQ2 and KCNQ3 form a heteromeric potassium ion channel known as the "M-channel." The M-channel is an important target for drug discovery since mutations in KCNQ2 and KCNQ3 are responsible for causing epilepsy (Biervert et al., 1998, *Science* 279:403-406; Singh et al., 1998, *Nature Genet.* 18:25-29; Schroeder et al., *Nature* 1998, 396:687-690). A screening program designed to identify activators or inhibitors of the M-channel would benefit greatly by the use of potassium channels comprising human IsK2 subunit protein as minus targets.

It is recognized that a drug discovery program aimed at identifying ion channel modulators should incorporate counterscreens that utilize the potassium channels responsible for the I_{Ks} current as minus targets. See, *e.g.*, Curran, 1998, *Current Opinion in Biotechnology* 9:565-572, at page 567, left column: "[S]creening

against I_{K_r} and I_{K_s} early in the drug development process should be considered.”

Since minK is a subunit of the potassium channel responsible for the I_{K_s} current (Sanguinetti et al., 1996, Nature 384:80-83) and since IsK2 is closely related to minK (see Figure 2 herein), then it follows that a drug discovery program aimed at
5 identifying ion channel modulators should incorporate counterscreens that utilize potassium channels containing IsK2 as minus targets.

The present invention also includes antibodies to the human IsK2 subunit protein. Such antibodies may be polyclonal antibodies or monoclonal antibodies. The antibodies of the present invention can be raised against the entire
10 human IsK2 subunit protein or against suitable antigenic fragments that are coupled to suitable carriers, *e.g.*, serum albumin or keyhole limpet hemocyanin, by methods well known in the art. Methods of identifying suitable antigenic fragments of a protein are known in the art. See, *e.g.*, Hopp & Woods, 1981, Proc. Natl. Acad. Sci. USA 78:3824-3828; and Jameson & Wolf, 1988, CABIOS (Computer Applications in the
15 Biosciences) 4:181-186.

For the production of polyclonal antibodies, human IsK2 subunit protein or antigenic fragments, coupled to a suitable carrier, are injected on a periodic basis into an appropriate non-human host animal such as, *e.g.*, rabbits, sheep, goats, rats, mice. The animals are bled periodically and sera obtained are tested for the
20 presence of antibodies to the injected subunit or antigen fragment. The injections can be intramuscular, intraperitoneal, subcutaneous, and the like, and can be accompanied with adjuvant.

For the production of monoclonal antibodies, human IsK2 subunit protein or antigenic fragments, coupled to a suitable carrier, are injected into an
25 appropriate non-human host animal as above for the production of polyclonal antibodies. In the case of monoclonal antibodies, the animal is generally a mouse. The animal's spleen cells are then immortalized, often by fusion with a myeloma cell, as described in Kohler & Milstein, 1975, Nature 256:495-497. For a fuller description of the production of monoclonal antibodies, see Antibodies: A Laboratory
30 Manual, Harlow & Lane, eds., Cold Spring Harbor Laboratory Press, 1988.

Gene therapy may be used to introduce human IsK2 subunit protein into the cells of target organs. Nucleotides encoding human IsK2 subunit protein can be ligated into viral vectors which mediate transfer of the nucleotides by infection of recipient cells. Suitable viral vectors include retrovirus, adenovirus, adeno-associated

virus, herpes virus, vaccinia virus, lentivirus, and polio virus based vectors.

Alternatively, nucleotides encoding human Isk2 subunit protein can be transferred into cells for gene therapy by non-viral techniques including receptor-mediated targeted transfer using ligand-nucleotide conjugates, lipofection, membrane fusion, or
5 direct microinjection. These procedures and variations thereof are suitable for *ex vivo* as well as *in vivo* gene therapy. Gene therapy with human Isk2 subunit protein will be particularly useful for the treatment of diseases where it is beneficial to elevate potassium channel activity.

The present invention includes processes for cloning orthologues of
10 human Isk2 subunit protein from non-human species. In general, such processes include preparing a PCR primer or a hybridization probe based upon SEQ.ID.NO.:1 that can be used to amplify a fragment containing the non-human Isk2 subunit (in the case of PCR) from a suitable DNA preparation or to select a cDNA or genomic clone containing the non-human Isk2 subunit from a suitable library. A preferred
15 embodiment of this process is a process for cloning the Isk2 subunit from mouse.

By providing DNA encoding mouse Isk2 subunit, the present invention allows for the generation of an animal model of human diseases in which Isk2 subunit activity is abnormal. Such animal models can be generated by making transgenic "knockout" or "knockin" mice containing altered Isk2 subunit genes.
20 Knockout mice can be generated in which portions of the mouse Isk2 subunit gene have been deleted. Knockin mice can be generated in which mutations that have been shown to lead to human disease are introduced into the mouse gene. Such knockout and knockin mice will be valuable tools in the study of the relationship between potassium channels and disease and will provide important model systems in which to
25 test potential pharmaceuticals or treatments for human diseases involving potassium channels.

Accordingly, the present invention includes a method of producing a transgenic mouse comprising:

- (a) designing PCR primers or an oligonucleotide probe based upon
30 SEQ.ID.NO.:1 for use in cloning the mouse Isk2 subunit gene or cDNA;
- (b) using the PCR primers or the oligonucleotide probe to clone at least a portion of the mouse Isk2 subunit gene or cDNA, the portion being large enough to use in making a transgenic mouse;

(c) producing a transgenic mouse having at least one copy of the mouse IsK2 subunit gene altered from its native state.

Methods of producing knockout and knockin mice are well known in the art. One method involves the use of gene-targeted ES cells in the generation of gene-targeted transgenic knockout mice and is described in, *e.g.*, Thomas et al., 1987, Cell 51:503-512, and is reviewed elsewhere (Frohman et al., 1989, Cell 56:145-147; Capecchi, 1989, Trends in Genet. 5:70-76; Baribault et al., 1989, Mol. Biol. Med. 6:481-492).

Techniques are available to inactivate or alter any genetic region to virtually any mutation desired by using targeted homologous recombination to insert specific changes into chromosomal genes. Generally, use is made of a "targeting vector," *i.e.*, a plasmid containing part of the genetic region it is desired to mutate. By virtue of the homology between this part of the genetic region on the plasmid and the corresponding genetic region on the chromosome, homologous recombination can be used to insert the plasmid into the genetic region, thus disrupting the genetic region. Usually, the targeting vector contains a selectable marker gene as well.

In comparison with homologous extrachromosomal recombination, which occurs at frequencies approaching 100%, homologous plasmid-chromosome recombination was originally reported to only be detected at frequencies between 10^{-6} and 10^{-3} (Lin et al., 1985, Proc. Natl. Acad. Sci. USA 82:1391-1395; Smithies et al., 1985, Nature 317: 230-234; Thomas et al., 1986, Cell 44:419-428). Nonhomologous plasmid-chromosome interactions are more frequent, occurring at levels 10^5 -fold (Lin et al., 1985, Proc. Natl. Acad. Sci. USA 82:1391-1395) to 10^2 -fold (Thomas et al., 1986, Cell 44:419-428) greater than comparable homologous insertion.

To overcome this low proportion of targeted recombination in murine ES cells, various strategies have been developed to detect or select rare homologous recombinants. One approach for detecting homologous alteration events uses the polymerase chain reaction (PCR) to screen pools of transformant cells for homologous insertion, followed by screening individual clones (Kim et al., 1988, Nucleic Acids Res. 16:8887-8903; Kim et al., 1991, Gene 103:227-233). Alternatively, a positive genetic selection approach has been developed in which a marker gene is constructed which will only be active if homologous insertion occurs, allowing these recombinants to be selected directly (Sedivy et al., 1989, Proc. Natl.

Acad. Sci. USA 86:227-231). One of the most powerful approaches developed for selecting homologous recombinants is the positive-negative selection (PNS) method developed for genes for which no direct selection of the alteration exists (Mansour et al., 1988, Nature 336:348-352; Capecchi, 1989, Science 244:1288-1292; Capecchi, 5 1989, Trends in Genet. 5:70-76). The PNS method is more efficient for targeting genes which are not expressed at high levels because the marker gene has its own promoter. Nonhomologous recombinants are selected against by using the Herpes Simplex virus thymidine kinase (HSV-TK) gene and selecting against its nonhomologous insertion with herpes drugs such as gancyclovir (GANC) or FIAU (1-10 (2-deoxy 2-fluoro-B-D-arabinofluranosyl)-5-iodouracil). By this counter-selection, the percentage of homologous recombinants in the surviving transformants can be increased.

Other methods of producing transgenic mice involve microinjecting the male pronuclei of fertilized eggs. Such methods are well known in the art.

15 The present invention includes a transgenic, non-human animal in which the animal's genome contains DNA encoding at least a portion of a human IsK2 subunit.

The following non-limiting examples are presented to better illustrate 20 the invention.

EXAMPLE 1

Identification of the human IsK2 subunits and cDNA cloning

DNA sequence encoding human minK was used to search the Genbank 25 database for homologous sequences. This search yielded an EST (AI246239) containing partial IsK2 sequence. This EST sequence was also found within the genomic DNA sequence AP00052 (nucleotides 80218-80589). From this information, synthetic oligonucleotide primers (SEQ.ID.Nos.:4 and 5) were used to amplify the full length coding region, some 5' untranslated sequence including an in 30 frame stop codon, and a small amount of 3' untranslated sequence from human genomic DNA (Clontech). This DNA fragment was subcloned into a DNA cloning vector (pCR2.1, Invitrogen) and sequenced with universal T7 and M13 reverse

sequencing primers using an ABI Prism 310 Genetic Analyzer. Based upon Northern analysis, which demonstrated IsK2 expression in human stomach, the same synthetic oligonucleotide primers (SEQ.ID.Nos.: 4 and 5) were used to amplify a cDNA fragment from human stomach mRNA. First strand stomach cDNA was synthesized from 1.5 µg human poly A+ mRNA (Clontech) using 19 µM random hexamer primers in 50 mM Tris pH 8.3, 8 mM MgCl₂, 3 mM KCl, 1 mM DTT, 2 mM dNTPs, and 24 units AMV Reverse Transcriptase at 42°C for 90 min. PCR was then carried out using 5% (2 µL) of the synthesized cDNA as the template in 20 mM Tris pH 8.75, 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% TritonX100, 0.1 mg/ml BSA, 200 µM dNTPs, 1 µM oligonucleotide primers, 5 Units Taq Plus Long (Stratagene). Cycling parameters were 25 cycles of 94°C for 1min, 56°C for 2min and 72°C for 3min. The cDNA fragment amplified in this manner was cloned into a TA cloning vector and sequenced. The sequences of the stomach cDNA and the genomic DNA fragments were identical.

15

EXAMPLE 2

Analysis of expression of human IsK2 subunits

RT-PCR Analysis: Transcription of the IsK2 gene in different tissues was examined by RT-PCR. cDNAs prepared from poly(A+)-RNA derived from different human tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testes, ovary, small intestine, colon, and peripheral blood leukocytes) were purchased from Clontech (Palo Alto, CA). The presence of IsK2 cDNA, demonstrating expression of this gene in a tissue, was assayed for by amplification of a fragment of it by PCR. Primers used were 5'-CCT CAT GGT GAT GAT TGG AA-3' (SEQ.ID.NO.:6) and 5' -GAG TGT TCC CGT CTC TTG GA-3' (SEQ.ID.NO.:7), amplifying an 84 bp fragment of IsK2. PCR amplification was carried out in a total volume of 20 µL containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 200 µM dNTPs, 250 nM of each oligonucleotide primer, 0.05 U Amplitaq Gold (Perkin Elmer Corp, Foster City, CA) and 0.4 ng cDNA. Cycling parameters consisted of 10 min at 94°C, followed by 35 cycles at 94°C for 30 sec, 56°C for 30 sec, and 72°C for 90 sec, and a final 7 min extension period at 72°C. Amplification products were analyzed by agarose gel electrophoresis.

Northern blot analysis: Northern blots of poly(A+)-RNA isolated from human heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testes, ovary, small intestine, colon, peripheral blood leukocytes, adrenal medulla, thyroid, adrenal cortex, stomach, uterus, and bladder were purchased from Clontech (Palo Alto, Ca.) and probed with ³²P-labeled, randomly primed IsK2 (SEQ.ID.:1). The hybridization was carried out in 5X SSPE, 10X Denhardt's solution, 50% formamide, 2% SDS, 100 µg/mL salmon sperm DNA at 42°C overnight. Blots were washed stepwise in 2X SSC, 0.05% SDS at 42°C for 40 minutes, followed by 1X SSC, 0.05% SDS at 50°C for 40 minutes. High stringency washes were carried out at 0.1X SSC, 0.05% SDS at 65°C for 40 minutes. Hybridization was detected either by exposure of the blots to Kodak XAR X-ray film or by electronic detection using a Molecular Dynamics phosphorimager.

EXAMPLE 3

15 Northern analysis to determine which alpha subunits might potentially interact with IsK2

Northern blot analysis was used to determine whether known α subunit genes are expressed in the same tissues as IsK2 and might, therefore, coassemble with it to form functional channels. Human multiple tissue Northern blots, containing 2 µg of poly A+ RNA isolated from skeletal muscle, uterus, colon, small intestine, bladder, heart, stomach, and prostate, were purchased from Clontech.. The blots were probed with probes specific for KvLQT1 (Genbank Accession # AF000571; nucleotides 1289-2147), KCNQ2 (Genbank Accession no. AF033348; nucleotides 1235-1420), KCNQ3 (Genbank Accession no. AF033347; nucleotides 1478-2482), KCNQ4 (Genbank Accession no. AF105202; nucleotides 1192-1360), and h-erg. (Genbank Accession no. U04270; nucleotides 178-3663). Probes were either cDNAs (isolated as restriction fragments and labeled with ³²P by random hexamer priming to a specific activity of > 1x10⁹cpm/µg) or synthetic oligonucleotide probes (labeled by fill in reactions to a specific activity of > 1x10⁸cpm/pmol). For blots probed with cDNA fragments, hybridization was carried out in 5X SSPE, 10X Denhardt's solution, 50% Formamide, 2% SDS, 100 µg/ml salmon sperm DNA at 42°C overnight. For blots probed with oligonucleotide

probes, hybridization was carried out in 5X SSPE, 2X Denhardt's solution, 0.5% SDS, 100 µg/ml at 42°C overnight. All blots were washed stepwise in 2X SSC, 0.05% SDS at 42°C for 40 minutes, followed by 1X SSC, 0.05% SDS at 50°C for 40 minutes. High stringency washes were carried out at 0.1X SSC, 0.05% SDS at 65°C for 40 minutes. Hybridization was detected either by exposures of the blots to Kodak XAR X-ray film or by electronic detection using a Molecular Dynamics phosphoimager. KvLQT1 is abundantly expressed in human stomach; KCNQ4 and h-erg are also expressed in human stomach, albeit at lower levels. These three proteins are, therefore, candidates for K channel α subunits that may coassemble with IsK2 to form functional potassium channels. See Figure 4 for results.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

WHAT IS CLAIMED IS:

1. An isolated DNA comprising nucleotides encoding a human IsK2 subunit protein.

5

2. The DNA of claim 1 comprising nucleotides encoding a polypeptide having the amino acid sequence shown in SEQ.ID.NO.:2.

3. The DNA of claim 1 comprising a nucleotide sequence selected from the group consisting of: SEQ.ID.NO.:1 and positions 79-447 of SEQ.ID.NO.:1.

10

4. An isolated DNA that hybridizes under stringent conditions to the DNA of claim 3.

15

5. An expression vector comprising the DNA of claim 3.

6. A recombinant host cell comprising the DNA of claim 3.

20

7. An isolated human IsK2 subunit protein.

8. The protein of claim 7 having the amino acid sequence shown in SEQ.ID.NO.: 2.

25

9. The protein of claim 8 containing a single amino acid substitution.

10. The protein of claim 8 containing two or more amino acid substitutions where the amino acid substitutions do not occur in conserved positions.

30

11. An antibody that binds specifically to a human IsK2 subunit protein.

12. A DNA or RNA oligonucleotide probe comprising at least 10 contiguous nucleotides from SEQ.ID.NO.:1.

13. A method for identifying substances that bind to potassium channels containing human IsK2 subunit protein comprising:

- (a) providing cells expressing a potassium channel containing human IsK2 subunit protein;
 - (b) exposing the cells to a substance that is not known to bind potassium channels containing human IsK2 subunit protein;
 - (c) determining the amount of binding of the substance to the cells;
 - (d) comparing the amount of binding in step (c) to the amount of binding of the substance to control cells where the control cells are substantially identical to the cells of step (a) except that the control cells do not express human IsK2 subunit protein;
- where if the amount of binding in step (c) is greater than the amount of binding of the substance to control cells, then the substance binds to potassium channels containing human IsK2 subunit protein.

14. A method of identifying substances that bind potassium channels containing human IsK2 subunit protein and thus are likely to be inhibitors or activators of potassium channels containing human IsK2 subunit protein comprising:

- (a) providing cells expressing potassium channels containing human IsK2 subunit protein;
- (b) exposing the cells to a compound that is known to bind to the potassium channels containing human IsK2 subunit protein in the presence and in the absence of a substance not known to bind to potassium channels containing human IsK2 subunit protein;
- (c) determining the amount of binding of the compound to the cells in the presence and in the absence of a substance not known to bind to potassium channels containing human IsK2 subunit protein;

where if the amount of binding of the compound in the presence of the substance differs from the amount of binding in the absence of the substance, then the substance binds potassium channels containing human IsK2 subunit proteins and is

likely to be an inhibitor or activator of potassium channels containing human IsK2 subunit protein.

15. A method of identifying activators or inhibitors of potassium
5 channels containing human IsK2 subunit protein comprising:

- (a) recombinantly expressing human IsK2 subunit protein or mutant human IsK2 subunit protein in a host cell so that the recombinantly expressed human IsK2 subunit protein forms potassium channels either by itself or by forming heteromers with other potassium channel subunit proteins;
- 10 (b) measuring the biological activity of the potassium channels formed in step (a) in the presence and in the absence of a substance suspected of being an activator or an inhibitor of potassium channels containing human IsK2 subunit protein;

where a change in the biological activity of the potassium channels
15 formed in step (a) in the presence as compared to the absence of the substance indicates that the substance is an activator or an inhibitor of potassium channels containing human IsK2 subunit protein.

1 ATAGCCAAAT CCAGAAAAGA TCCGTTTTCC TAACCTTGTT CGCCTATTTT ATTATTTAAA
61 TTGCAGCAGG AGGGAAGCAT GTCTACTTTA TCCAATTTCA CACAGACGCT GGAAGACGTC
121 TTCCGAAGGA TTTTATTAC TTATATGGAC AATTGGCGCC AGAACACAAC AGCTGAGCAA
181 GAGGCCCTCC AAGCCAAAGT TGATGCTGAG AACTTCTACT ATGTCATCCT GTACCTCATG
241 GTGATGATTG GAATGTTCTC TTTCATCATC GTGGCCATCC TGGTGAGCAC TGTGAAATCC
301 AAGAGACGGG AACACTCCAA TGACCCCTAC CACCAGTACA TTGTAGAGGA CTGGCAGGAA
361 AAGTACAAGA GCCAAATCTT GAATCTAGAA GAATCGAAGG CCACCATCCA TGAGAACATT
421 GGTGCGGCTG GGTTCAAAAT GTCCCCCTGA TAAGGGAGAA AGGCACCAAG C

FIG. 1A

1 MSTLSNFTQT LEDVFRRIFI TYMDNWRQNT TAEQEALQAK VDAENFYVI LYLMVMIGMF
61 SFIIVAILVS TVKSKRREHS NDPYHQYIVE DWQEKYKSI LNLEESKATI HENIGAAGFK
121 MSP

FIG. 1B

ISK2
MSTLSN...FTQTL.....EDVFR.....RIFITYMDNWRQNTTAEQEALQAKVDAE
MINK
M.ILSNTTAVTPFLTKLWQETVQQGGNMSGGLARRSPRSGD.....GKLEAL.....
consensus M..LSN T L E V R D EAL

ISK2
NFYYVILYLMVMIGMFSFIIIVAILVSTVKSRRHSNDPYHQYIVED.WQEKYKS..QIL
MINK
...YV...LMVL.GFFGFFTLGIMLSYIRSKKLEHSNDPFNVYIESDAWQEKDKAYVQAR
consensus YV LMV G F F I S SK EHSNDP YI D WQEK K Q

ISK2 NLE..ESKATIHENIGAAG.....FKMSP
MINK VLESYRS.CYVVENHLAIEQPNTLHPETKKPSP
consensus LE S EN A K SP

FIG.2

FIG.3A

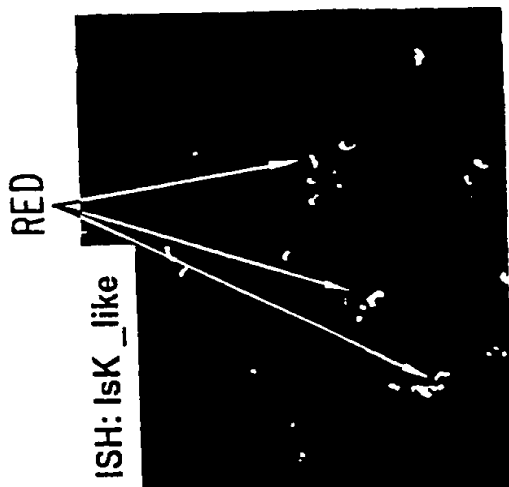


FIG.3B

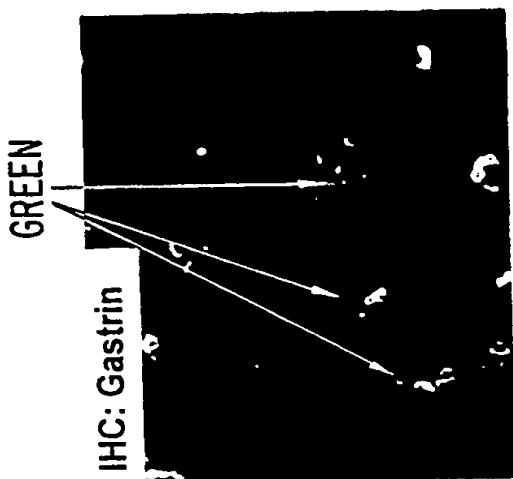


FIG.3C

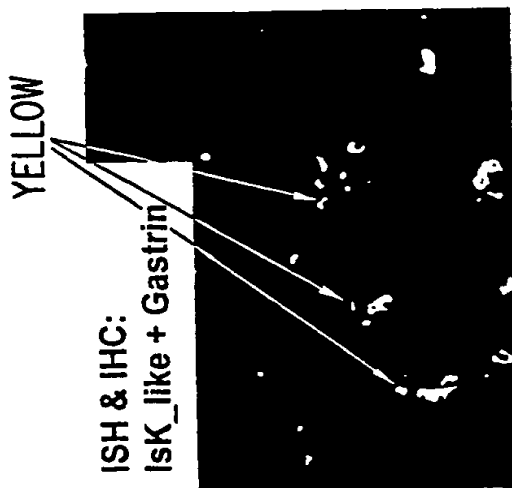


FIG.3D

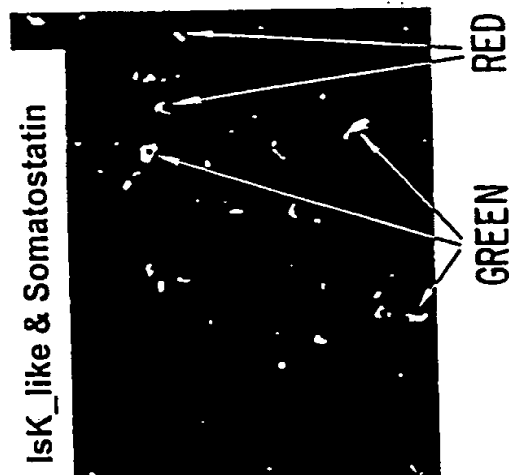


FIG.3E

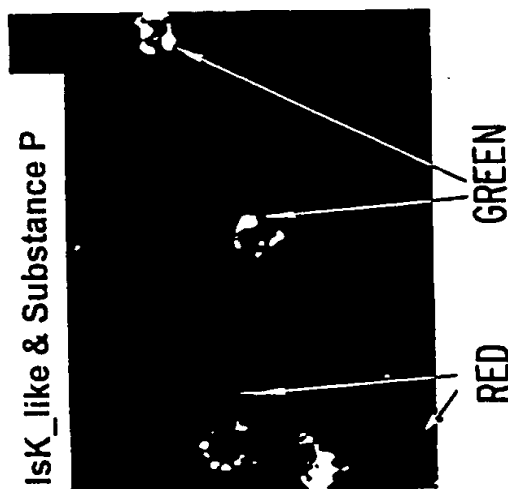
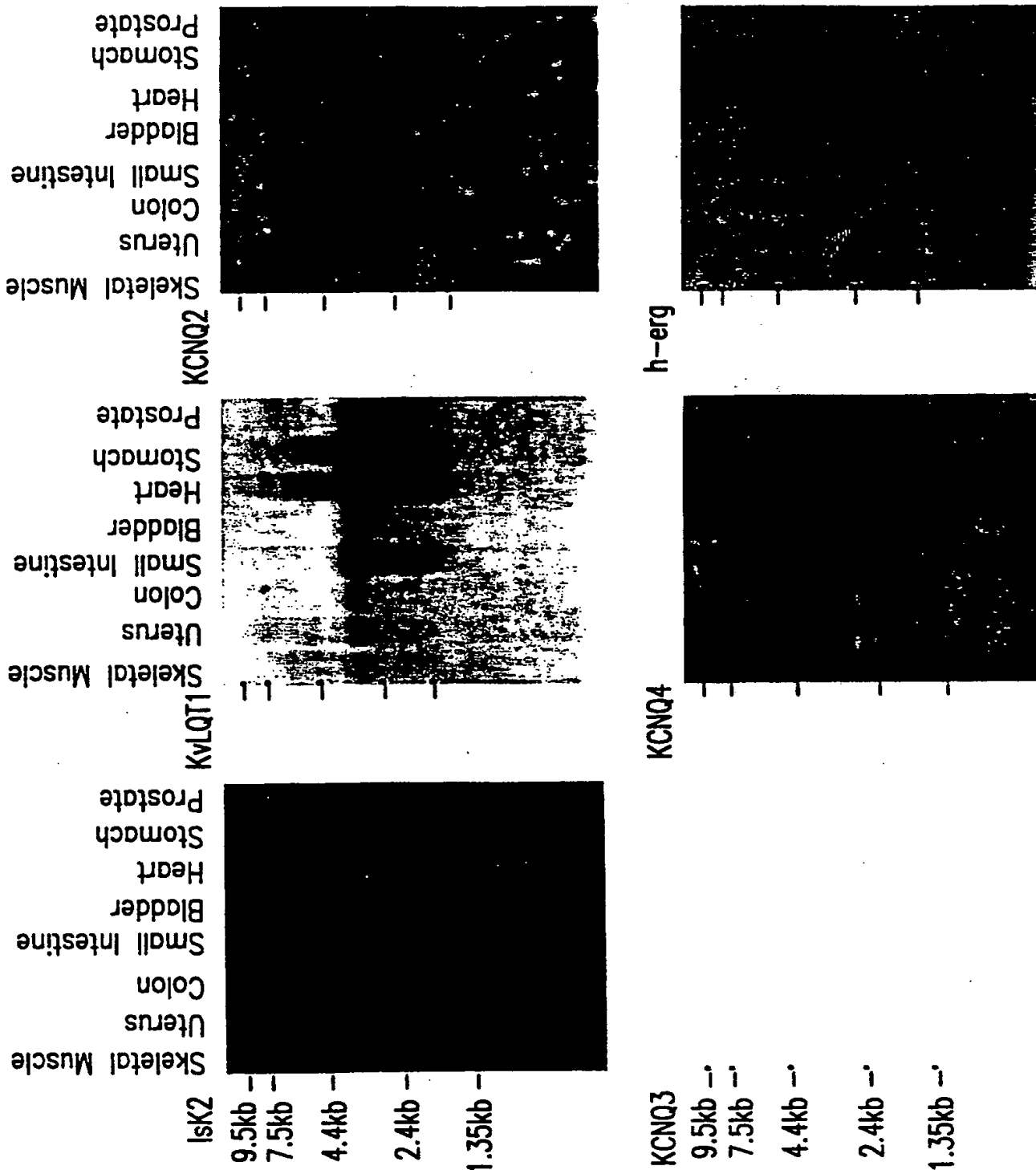


FIG.3F

FIG. 4



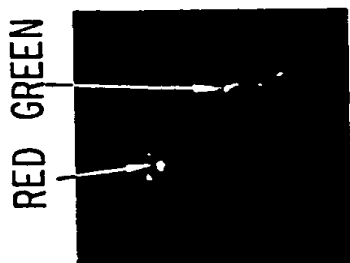


FIG. 5A

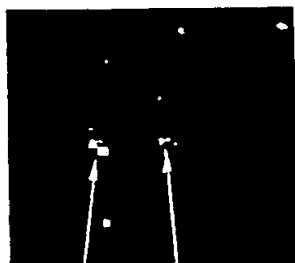


FIG. 5B

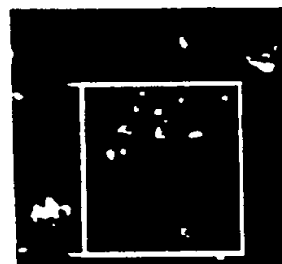


FIG. 5C

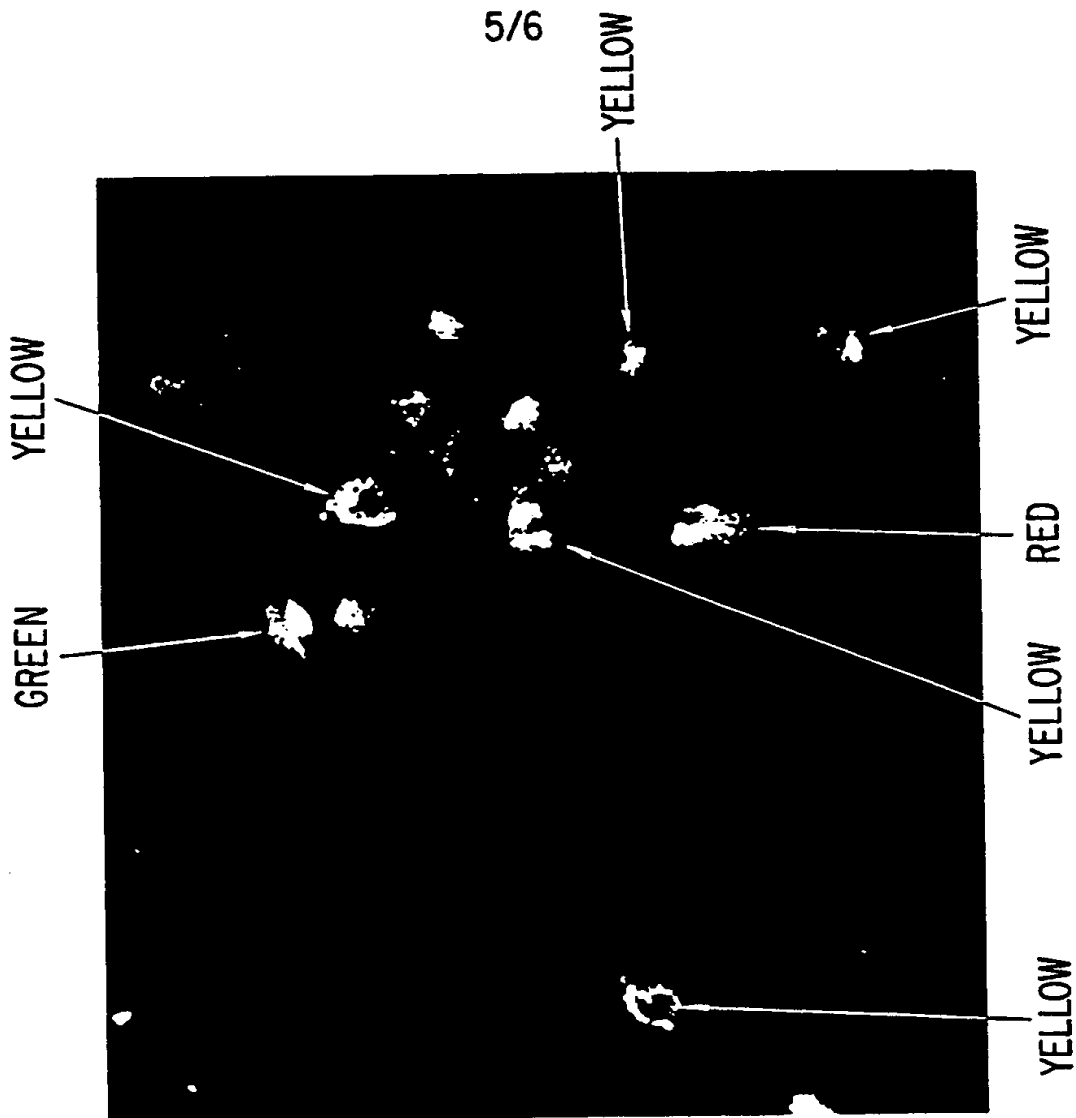


FIG. 5D

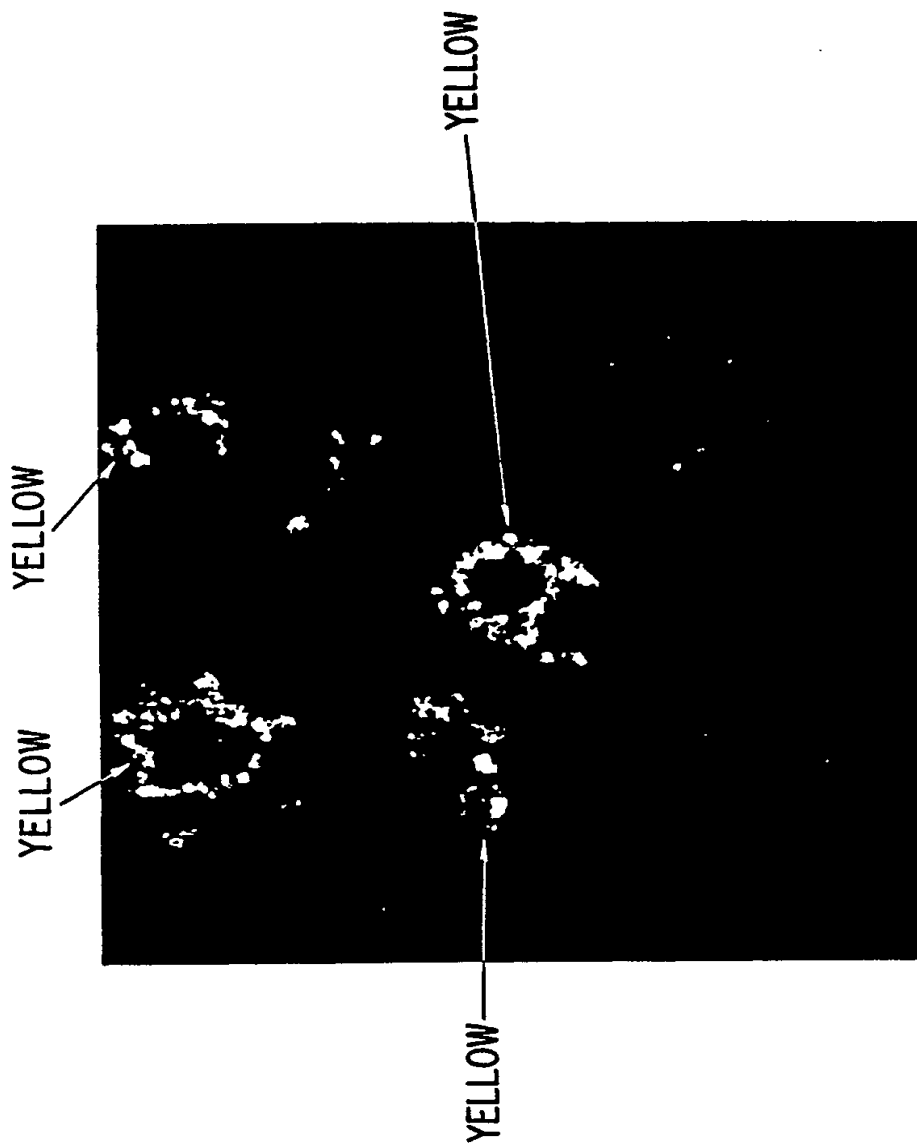


FIG.5E

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/28014**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350, 387.1, 387.9; 536/23.1, 23.5; 435/69.1, 71.1, 71.2, 325, 471, 252.3, 254.11, 320.1, 7.1, 7.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — A	ABBOTT et al. MiRP1 Forms IKr Potassium Channels with HERG and is Associated with Cardiac Arrhythmia. Cell. 16 December 1999, Vol. 97, pages 175-287, especially page 185, column 2 and page 176, Figure 1.	1-8, 12 — 9-11, 13-15

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents: *A* document defining the general state of the art which is not considered to be of particular relevance *B* earlier document published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed		*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art *A* document member of the same patent family
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Date of the actual completion of the international search

09 JANUARY 2001

Date of mailing of the international search report

21 MAR 2001

 Name and mailing address of the ISA/US
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/28014

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (7):

C12N 5/10, 15/12, 15/63, 15/64; C07K 14/705, 14/71, 16/18; G01N 33/53, 33/567

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

530/350, 387.1, 387.9; 536/23.1, 23.5; 435/69.1, 71.1, 71.2, 325, 471, 252.3, 254.11, 320.1, 7.1, 7.2

0/502